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REMARKS/ARGUMENTS

Claims 1, 3-6, 10-17, 19-22, 26-29 and 39 are active in this application. Support for the amendment to Claims 13-15, 17 and 27 is found on page 5, lines 19-23 and page 9, line 9. Further support for the amendment to Claim 27 is found on page 9, lines 21-31. Support for Claim 39 is found in Claim 1 and page 4, lines 21-22. The specification has been amended to include the date of deposit and the full address of the depository. Support for this amendment is found in the deposit receipts which were made of record when the application was filed. Copies of the deposit receipts for DSM 13457 are attached. No new matter is added.

Applicants thank the Examiner for indicating that Claims 1, 3-6, 10, 11, 19, 20, 22 and 26 are allowed. Applicants also thank the Examiner for the helpful discussion granted to the Applicants' representative. During this discussion the enablement rejections were discussed. The substance of this discussion is summarized and expanded upon in the remarks submitted herein.

In view of the amendments submitted herein and the following remarks, Applicants request allowance of all pending claims.

The rejection of Claims 13-15, 17 and 27 under 35 U.S.C. § 112, first paragraph ("enablement") is respectfully traversed.

The Applicants have previously noted why the specification enables the polynucleotides that are structurally related to SEQ ID NO:1 which also encode a protein with OxyR transcriptional regulation activity. During the above-noted discussion, the Examiner indicated that the rejection would be reconsidered if Applicants provided evidence pointing to the fact that the structural features of oxyR that are important for oxidation, transcriptional regulation and DNA binding are known. Such evidence is already of record and is again pointed to herein. Specifically, Applicants direct the Examiner's attention to Kullik, I., et al., *J.Bacteriol.* 177:1275-1284 ("Kullik I"); and Kullik, I., et al., *J.Bacteriol.*

177:1285-1291 (“Kullik II”). For convenience, copies of these publications are attached hereto. Kullik I describe regions important for oxidation and transcription activation whereas Kullik II describe regions important for DNA binding and multimerization. In view of this information and Applicants’ remarks previously made of record, Applicants request withdrawal of this rejection.

The rejection of Claim 27-28 under 35 U.S.C. § 112, first paragraph (“enablement”) is respectfully traversed.

Claim 27 has been amended to include the way in which overexpression is achieved- “by increasing the copy number of said polynucleotide or operably linking a promoter to said polynucleotide.” This, as noted above, is described on page 9, lines 21-31. Accordingly, it would not require undue experimentation to make and/or use the *Corynebacterium* claimed in Claims 27 and 28.

Withdrawal of this ground of rejection is requested.

The rejection of Claim 29 under 35 U.S.C. § 112, first paragraph (“enablement”) is respectfully traversed.

Corynebacterium glutamicum DSM 13457 claimed in Claim 29 has been deposited under the terms of the Budapest Treaty as described on page 14, line 11 and further evidenced by the deposit receipt made of record when the present application was filed. For reference, a copy of that deposit receipt is attached hereto. The specification on page 14 has been amended consistent with the Examiner’s suggestions on page 8 of the Office Action. As the requisite assurances concerning the deposited material has already been made of record, Applicants request that this ground of rejection be withdrawn.

The rejection of Claim 16 under 35 U.S.C. § 112, first paragraph is respectfully traversed.

As noted in Applicants' previous response, support for the fragments in this claim is found on page 5 of the application as originally filed. In this portion of specification, the fragments can be used to identify and/or isolate DNAs that encode the OxyR transcriptional regulator. One such method employing such fragments or primers is in the polymerase chain reaction. It is common in the field to amplify a coding region of a gene using primers that lie just outside of the coding region, i.e., in the non-coding regions both 5' and 3' to the coding region. This is typically performed to ensure amplification of the entire coding sequence of the gene. Accordingly, one would clearly recognize that fragments of SEQ ID NO:1 within the coding region as well as in non-coding portions were envisioned and described by the Applicants for the reasons set forth above.

Withdrawal of this ground of rejection is requested.

The rejection of Claim 16 under 35 U.S.C. § 112, second paragraph is addressed by amendment. The fragments of the complement of SEQ ID NO:1 have been removed from the claim since the Examiner deems that SEQ ID NO:1 is double stranded. Withdrawal of this ground of rejection is requested.

The objection to Claim 12 under 37 CFR 1.75(c) and the objection to Claims 21 and 27-28 are addressed by amendment.

Applicants request allowance of this application. Early notice of such is requested.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Norman F. Oblon



Daniel J. Pereira, Ph.D.

Attorney of Record

Registration No. 45,518

Customer Number

22850

Tel: (703) 413-3000

Fax: (703) 413 -2220

(OSMMN 06/04)


BUDAPESTER VERTRAG ÜBER DIE INTERNATIONALE
ANERKENNUNG DER HINTERLEGUNG VON MIKROORGANISMEN
FÜR DIE ZWECKE VON PATENTVERFAHREN

INTERNATIONALES FORMBLATT

Degussa Hüls AG
Kantstr. 2

33790 Halle/Künsebeck

EMPFANGSBESTÄTIGUNG BEI ERSTHINTERLEGUNG,
ausgestellt gemäß Regel 7.1 von der unten angegebenen
INTERNATIONALEN HINTERLEGUNGSSTELLE

I. KENNZEICHNUNG DES MIKROORGANISMUS	
Vom HINTERLEGER zugeteiltes Bezugszeichen: DSM 5715/pT-oxyRexp	Von der INTERNATIONALEN HINTERLEGUNGSSTELLE zugeteilte EINGANGSNUMMER: DSM 13457
II. WISSENSCHAFTLICHE BESCHREIBUNG UND/ODER VORGESCHLAGENE TAXONOMISCHE BEZEICHNUNG	
Mit dem unter I. bezeichneten Mikroorganismus wurde (X) eine wissenschaftliche Beschreibung (X) eine vorgeschlagene taxonomische Bezeichnung eingereicht. (Zutreffendes ankreuzen).	
III. EINGANG UND ANNAHME	
Diese internationale Hinterlegungsstelle nimmt den unter I bezeichneten Mikroorganismus an, der bei ihr am 2000-04-17 (Datum der Ersthinterlegung) ¹ eingegangen ist.	
IV. EINGANG DES ANTRAGS AUF UMWANDLUNG	
Der unter I bezeichnete Mikroorganismus ist bei dieser Internationalen Hinterlegungsstelle am eingegangen (Datum der Ersthinterlegung) und ein Antrag auf Umwandlung dieser Ersthinterlegung in eine Hinterlegung gemäß Budapest Vertrag ist am eingegangen (Datum des Eingangs des Antrags auf Umwandlung).	
V. INTERNATIONALE HINTERLEGUNGSSTELLE	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Anschrift: Mascheroder Weg 1b D-38124 Braunschweig	Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten:  Datum: 2000-05-04

¹ Falls Regel 6.4 Buchstabe d zutrifft, ist dies der Zeitpunkt, zu dem der Status einer internationalen Hinterlegungsstelle erworben worden ist.


BUDAPESTER VERTRAG ÜBER DIE INTERNATIONALE
ANERKENNUNG DER HINTERLEGUNG VON MIKROORGANISMEN
FÜR DIE ZWECKE VON PATENTVERFAHREN

INTERNATIONALES FORMBLATT

Degussa Hüls AG
Kantstr. 2

33790 Halle/Künsebeck

LEBENSFÄHIGKEITSBESCHEINIGUNG
ausgestellt gemäß Regel 10.2 von der unten angegebenen
INTERNATIONALEN HINTERLEGUNGSSTELLE

I. HINTERLEGER	II. KENNZEICHNUNG DES MIKROORGANISMUS
Name: Degussa Hüls AG Kantstr. 2 Anschrift: 33790 Halle/Künsebeck	Von der INTERNATIONALEN HINTERLEGUNGSSTELLE zugeteilte EINGANGSNUMMER: DSM 13457 Datum der Hinterlegung oder Weiterleitung ¹ : 2000-04-17
III. LEBENSFÄHIGKEITSBESCHEINIGUNG	
Die Lebensfähigkeit des unter II genannten Mikroorganismus ist am 2000-04-17 ² geprüft worden. Zu diesem Zeitpunkt war der Mikroorganismus (X) ³ lebensfähig () ³ nicht mehr lebensfähig	
IV. BEDINGUNGEN, UNTER DENEN DIE LEBENSFÄHIGKEITSPRÜFUNG DURCHGEFÜHRT WORDEN IST⁴	
V. INTERNATIONALE HINTERLEGUNGSSTELLE	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Anschrift: Mascheroder Weg 1b D-38124 Braunschweig	Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten:  Datum: 2000-05-04

¹ Angabe des Datums der Ersthinterlegung. Wenn eine erneute Hinterlegung oder eine Weiterleitung vorgenommen worden ist, Angabe des Datums der jeweils letzten erneuten Hinterlegung oder Weiterleitung.

² In den in Regel 10.2 Buchstabe a Ziffer ii und iii vorgesehenen Fällen Angabe der letzten Lebensfähigkeitsprüfung.

³ Zutreffendes ankreuzen.

⁴ Ausfüllen, wenn die Angaben beantragt worden sind und wenn die Ergebnisse der Prüfung negativ waren.


BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa Hüls AG
Kantstr. 2

33790 Halle/Künsebeck

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: DSM 5715/pT-oxyRexp	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13457
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I. above was accompanied by:</p> <p>(X) a scientific description (X) a proposed taxonomic designation</p> <p>(Mark with a cross where applicable).</p>	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2000-04-17 (Date of the original deposit) ¹ .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2000-05-04

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE


INTERNATIONAL FORM

Degussa Hüls AG
Kantstr. 2

33790 Halle/Künsebeck

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa Hüls AG Kantstr. 2 Address: 33790 Halle/Künsebeck	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13457 Date of the deposit or the transfer ¹ : 2000-04-17
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2000-04-17 ² . On that date, the said microorganism was (X) ³ viable () ³ no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2000-05-04

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

Mutational Analysis of the Redox-Sensitive Transcriptional Regulator OxyR: Regions Important for Oxidation and Transcriptional Activation

INES KULLIK,^{1†} MICHEL B. TOLEDANO,^{1‡} LOUIS A. TARTAGLIA,^{2§} AND GISELA STORZ^{1*}

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, Bethesda, Maryland 20892,¹ and Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720²

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OxyR is a redox-sensitive transcriptional regulator of the LysR family which activates the expression of genes important for the defense against hydrogen peroxide in *Escherichia coli* and *Salmonella typhimurium*. OxyR is sensitive to oxidation and reduction, and only oxidized OxyR is able to activate transcription of its target genes. Using site-directed mutagenesis, we found that one cysteine residue (C-199) is critical for the redox sensitivity of OxyR, and a C-199→S mutation appears to lock the OxyR protein in the reduced form. We also used a random mutagenesis approach to isolate eight constitutively active mutants. All of the mutations are located in the C-terminal half of the protein, and four of the mutations map near the critical C-199 residue. In vivo as well as in vitro transcription experiments showed that the constitutive mutant proteins were able to activate transcription under both oxidizing and reducing conditions, and DNase I footprints showed that this activation is due to the ability of the mutant proteins to induce cooperative binding of RNA polymerase. Unexpectedly, RNA polymerase was also found to reciprocally affect OxyR binding.

The OxyR protein is a transcriptional activator of genes important for the defense against oxidative stress in *Escherichia coli* and *Salmonella typhimurium* (14, 37). Upon exposure to hydrogen peroxide, OxyR induces the expression of several genes, including *katG* (encoding HPI catalase), *ahpCF* (encoding an alkyl hydroperoxide reductase), *dps* (encoding a nonspecific DNA-binding protein), *gorA* (encoding glutathione reductase), and *oxyS* (encoding a small untranslated, regulatory RNA), and the cells become more resistant to oxidative stress (3, 4, 39, 41). During normal growth, OxyR also acts as a repressor, negatively autoregulating its own expression and the expression of the Mu phage *mor* gene (6, 14, 38). Here and in the accompanying paper (20), we identify regions of the OxyR protein critical for activation and repression.

OxyR is 34 kDa in size and belongs to the LysR-type family of transcriptional regulators (6, 14, 37, 43). LysR family members are all DNA-binding proteins which positively regulate expression of their target genes, and many of the regulators also repress their own expression (reviewed in reference 29). The ability of most LysR-type proteins to activate transcription is dependent on the presence of a coinducer, such as octopine for the *Agrobacterium tumefaciens* OccR protein (42), indole-glycerol phosphate for *Pseudomonas aeruginosa* TrpI (12), *N*-acetylserine for *E. coli* CysB (26), and flavonoids for the NodD proteins of different species of *Rhizobium* (reviewed in reference 16). The central region of the LysR-type proteins is assumed to be involved in coinducer binding, since *Rhizobium*

leguminosarum NodD and *Pseudomonas putida* NahR mutations which caused an altered response to the inducer mapped to the central domain (9, 18, 24, 30), and an exchange between the central domains of NodD proteins from different *Rhizobium* species leads to a different coinducer specificity of the resulting hybrid proteins (31). The fact that members of the LysR-type family of transcriptional regulators have only little homology in this protein region probably reflects the variety of coinducers to which these proteins respond. Unlike many of the other LysR-type regulators, the OxyR protein does not appear to bind a coinducer but rather is activated by direct oxidation. In vitro transcription experiments with purified components showed that the oxidized but not the reduced form of OxyR was able to activate transcription (35), but the redox-active center has not been characterized.

A transcriptional activator may increase RNA polymerase binding, open complex formation, or promoter clearance (reviewed in reference 2). A few studies suggest that LysR-type regulators contact the α subunit of RNA polymerase and act to increase polymerase binding to the promoter. OxyR binds adjacent to the -35 sequence of the positively regulated promoters, and Tao et al. have shown that OxyR acts cooperatively to increase RNA polymerase binding to the *katG* promoter (36). Mutations in the α subunit of polymerase, such as a change of R-265 to C (R265C), prevented activation by OxyR (36). Similarly, mutations at codon 271 of the α subunit of RNA polymerase prevent activation by the LysR-type regulator CysB (40). The domains in OxyR and other LysR family members that touch RNA polymerase have not been identified.

In recent studies on the DNA binding properties of OxyR, we found that the reduced and oxidized (activated) forms of OxyR require different DNA contacts for binding (41). Oxidized OxyR binds in four adjacent major grooves, while reduced OxyR contacts two pairs of adjacent major grooves separated by one helical turn. The reduced protein has significantly reduced affinities for the *katG* and *ahpCF* promoters, showing that only a subset of the OxyR-regulated promoters

* Corresponding author. Mailing address: National Institutes of Health, Building 18T, Room 101, Bethesda, MD 20892-5430. Phone: (301) 402-0968. Fax: (301) 402-0078. Electronic mail address: storz@helix.nih.gov.

† Present address: Institut für Medizinische Mikrobiologie, Universität Zürich, CH-8028 Zürich, Switzerland.

‡ Present address: Department of Pharmacology and Toxicology, Rutgers University College of Pharmacy, Piscataway, NJ 08855-1179.

§ Present address: Millennium Inc., Cambridge, MA 02139.

carry determinants for the binding of reduced OxyR. The two binding modes probably allow OxyR to repress the *oxyR* and *mom* promoters during normal growth, while activating *kaiG*, *ahpCF*, *dps*, *gorA*, and *oxyS* only upon oxidative stress. These studies also suggest that the conformations of reduced and oxidized OxyR are significantly different.

In the present study, we used site-directed as well as random mutagenesis to characterize the domains of the OxyR protein required to sense oxidative stress and affect transcription initiation. The six possible redox-reactive cysteines in OxyR were mutagenized to serine, and a single cysteine (Cys-199) was found to be critical for activity. Random mutagenesis revealed that a region around this critical cysteine is involved in transcriptional activation, since several mutations causing the constitutive phenotype mapped to this region. We were also able to show that the ability of mutants to activate transcription correlates with their ability to induce RNA polymerase binding. Moreover, DNase I footprinting assays demonstrated that while OxyR induces polymerase binding, RNA polymerase also reciprocally influences OxyR binding.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are described in Table 1. Strain GSO5 was constructed as follows. The 0.2-kb *HindIII*-*SplI* fragment of pAQ17 (14), carrying the *oxyR*-*oxyS* promoter region, was cloned into the unique *SmaI* site of pTS7 (1) to create the *galk* fusion. The plasmid was then recombined onto λ Y2053 (cl857 *galk* *nin5*) and integrated into the *attA* site of SA2692 (1). A Δ *oxyR::kan* deletion-insertion mutation (3) was subsequently moved into the strain by P1 transduction. pAQ5 (34) was used for the random mutagenesis and is a pACYC184 derivative which carries *oxyR* on a *Bam*HI-*EcoRV* fragment. Since these pACYC184-derived plasmids did not prove to be suitable for double-strand sequencing, the *Bam*HI-*HindIII* *oxyR* fragments were subcloned into M13mp18 for sequencing. For overproduction of the mutant OxyR proteins, *oxyR* was cloned behind the *P_{lac}* promoter of pKK177-3 as follows. First, the *SplI*-*HindIII* fragment of wild-type *oxyR* in pUC18 carrying the modified Shine-Dalgarno sequence (35) was replaced with the same fragment of the mutant *oxyR* genes from pACYC184. These constructs were sequenced, and subsequently the *EcoRI*-*HindIII* fragment was subcloned into pKK177-3. All sequencing and subcloning were carried out by standard procedures.

Media and growth conditions. Strains were grown in LB medium (25), and ampicillin (100 μ g/ml [final concentration]), kanamycin (25 μ g/ml), chloramphenicol (25 μ g/ml), or tetracycline (15 μ g/ml) was added when appropriate. For overproduction of the mutant proteins, the cells were grown in TB medium (28). The resistance of strains to hydrogen peroxide and cumene hydroperoxide was assayed by zones of inhibition, which were determined as described previously (13) except that the strains were grown in and plated on LB medium containing the appropriate antibiotics.

Mutagenesis. Site-directed mutagenesis was carried out with an oligonucleotide-directed in vitro mutagenesis system (Amersham, Arlington Heights, Ill.). The base pair substitutions that were introduced are listed in Table 2. Random mutagenesis with hydroxylamine was carried out with approximately 10 μ g of purified pAQ5 plasmid DNA as described previously (15). The DNA (20 μ l) was mixed with 100 μ l of a 0.5 M potassium phosphate-5 mM EDTA solution (pH 6) and 80 μ l of a freshly prepared 1 M hydroxylamine solution (pH 6) and incubated for 60 min at 65°C. The samples were dialyzed extensively against Tris-EDTA buffer and used directly (5- to 10- μ l aliquots) to transform *E. coli* XL1-blue cells. The XL1-blue transformants were rinsed off the plates, and the plasmid DNA was reisolated and then used to transform the GSO5 recipient strain. Finally, the GSO5 transformants were screened for the desired phenotype on MacConkey agar plates.

Protein overexpression and purification. For overproduction of the mutant OxyR proteins, *oxyR* deletion strains (TA4484) with the corresponding pKK177-3 derivatives were grown to mid-exponential phase and then induced with 250 μ g of IPTG (isopropyl- β -D-thiogalactopyranoside) per ml for 2 h. The cells were lysed by several passages through a French pressure cell, and the insoluble fraction was removed by centrifugation. The soluble fraction was then applied to a heparin-Sepharose column (Pharmacia, Piscataway, N.J.). The purification procedure was carried out as described previously (35) except that the buffer Z contained 50 mM HEPES (N-2-hydroxypiperazine-N'-2-ethanesulfonic acid) (pH 8), 5 mM MgCl₂, 0.5 mM EDTA (pH 8), and 10% (vol/vol) glycerol. The A233V and E225K mutants were eluted with a 0.1 to 0.4 M KCl gradient instead of the second wash with buffer Z containing 0.2 M KCl. Both the A233V and E225K mutants eluted at about 0.2 M KCl. Aliquots of the peak fractions were analyzed on sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) gels, using the buffer system of Laemmli (21). The protein

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
XL1-blue	F' [<i>proAB</i> ⁺ <i>lacI</i> ^s <i>lacZ</i> Δ M15 Tn10 (Tc ^r)]	8
D1210	HB101/F' [<i>lacI</i> ^s]	5
TA4112	<i>oxyR</i> Δ 3	13
TA4484	<i>oxyR</i> Δ 3, pMC7	39
SA2692	HB101 <i>recA</i> ⁺ Δ <i>lac</i> Δ <i>gal</i> -165	1
GSO5	SA2692 Δ <i>oxyR::kan</i> (λ Y2053 <i>oxyS-galk</i>)	This study
Plasmids		
pTS7	pBR322 <i>int</i> P'P; promoterless <i>lacZ</i> and <i>galETK</i> , Ap ^r	1
pMC7	<i>lacI</i> ^s Tc ^r	10
pACYC184	Cm ^r Tc ^r	11
pKK177-3	<i>P_{lac}</i> promoter, derivative of pKK223-3, Ap ^r	7
pAQ5	<i>oxyR</i> wt ^a in pACYC184, Cm ^r , Ap ^r	34
pAQ17	<i>oxyR</i> wt in pUC12	14
pAQ25	<i>oxyR</i> wt in pKK177-3 with altered SD ^b sequence, Ap ^r	35
pGSO8	<i>oxyR</i> C25S in pUC18	This study
pGSO9	<i>oxyR</i> C143S in pUC18	This study
pGSO10	<i>oxyR</i> C180S in pUC18	This study
pGSO11	<i>oxyR</i> C208S in pUC18	This study
pGSO12	<i>oxyR</i> C259S in pUC18	This study
pGSO13	<i>oxyR</i> 5CS ^c in pUC18	This study
pGSO51	<i>oxyR</i> T100I in pACYC184	This study
pGSO52	<i>oxyR</i> H114Y in pACYC184	This study
pGSO53	<i>oxyR</i> H198Y in pACYC184	This study
pGSO54	<i>oxyR</i> H198R in pACYC184	This study
pGSO55	<i>oxyR</i> R201C in pACYC184	This study
pGSO56	<i>oxyR</i> C208Y in pACYC184	This study
pGSO58	<i>oxyR</i> A233V in pACYC184	This study
pGSO59	<i>oxyR</i> A233T in pACYC184	This study
pGSO60	<i>oxyR</i> G253K in pACYC184	This study
pGSO66	<i>oxyR</i> E225K in pACYC184	20
pGSO67	<i>oxyR</i> H198R in pKK177-3	This study
pGSO68	<i>oxyR</i> C199S in pKK177-3	This study
pGSO69	<i>oxyR</i> A233V in pKK177-3	This study
pGSO70	<i>oxyR</i> E225K in pKK177-3	This study
pGSO71	<i>oxyR</i> S33N in pKK177-3	This study
pGSO72	<i>oxyR</i> C199S in pUC18	This study
pGSO73	<i>oxyR</i> C199S in pACYC184	This study

^a wt, wild type.

^b SD, Shine-Dalgarno.

^c Cysteine residues 25, 143, 180, 208, and 259 changed to serine.

concentration in all of the preparations was approximately 0.8 mg/ml, and roughly 8 mg of approximately 90% pure OxyR protein was obtained from a 1-liter cell culture.

Primer extension assays. Cells were grown to an optical density at 600 nm of 0.4, and then half of each sample was treated with hydrogen peroxide (200 μ M final concentration) for 10 min. Total RNA was isolated by using hot phenol, and 0.1 pmol of an end-labeled *oxyS* oligonucleotide (5'-GCAAAAGTTCACGT TGG) was annealed to 3 μ g of total RNA as described previously (33 [short protocol]). The extension reaction was performed with Superscript reverse transcriptase from Gibco BRL (Gaithersburg, Md.) in the reaction buffer provided. The extension products were separated on an 8% sequencing gel and were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

DNase I footprinting assays. An end-labeled DNA fragment (20,000 cpm) was incubated with 1 to 2 μ l of the soluble fraction of a crude cell extract or with 10 to 100 ng of pure OxyR protein in 25 μ l of 0.5 \times TM buffer (39). Aliquots (6 pmol) of RNA polymerase (Pharmacia) were added to the binding reaction mixtures when indicated. The binding reaction mixtures were then treated with DNase I as described previously (39). The crude cell lysates were prepared by sonicating the pellet of a 5-ml overnight culture in 800 μ l of 10 mM Tris buffer (pH 8) containing 20% glycerol. The soluble fraction was obtained by centrifugation.

In vitro transcription assays. The transcription assays were carried out at 37°C as described previously (35). Purified OxyR (5 pmol) was first incubated with

TABLE 2. Sensitivities of *oxyR* cysteine-to-serine mutants to oxidants

Strain	Codon exchange	Zone(s) of inhibition ^a (mm) with:		Phenotype ^b
		10% H ₂ O ₂	4% CHP	
Vector (pUC18)		36	27	
Wild type		24 (29)	18	
C25S	TGC→TCC	24 (29)	19	Wild type
C143S	TGC→TCC	24 (29)	18	Wild type
C180S	TGC→TCC	24 (29)	18	Wild type
C199S	TGT→TCT	40	26	Noninducible
C208S	TGT→TCT	29	18	Wild type
C259S	TGC→TCC	25 (31)	17	Wild type
SCS ^c		29	17	Wild type

^a Total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H₂O₂) or cumene hydroperoxide (CHP). The values are from a representative assay. The numbers in parentheses indicate the size of the second zone of partial growth.

^b Phenotype of mutants with respect to their sensitivity to oxidants.

^c In this mutant, cysteine residues 25, 143, 180, 208, and 259 were changed to serine.

pAQ17 (0.2 µg) for 10 min, and then RNA polymerase (6 pmol) was added and the reaction mixtures were incubated for another 10 min. After the addition of 1 µl of a 25 mM nucleoside triphosphate mixture, the reaction mixtures were incubated for an additional 5 min, and then the reactions were terminated by several phenol extractions. One-third of each sample was analyzed by primer extension with end-labeled *oxyS* (5'-GCAAAAGTTCACGTTGG) and *bla* (5'-AGGGCGACACGGAATGTTGAATACTCATA) oligonucleotide primers. The primer extension assays were carried out as described above with the exception that after the extension reaction, the samples were treated with 5 µg of RNase A and subjected to a phenol extraction and ethanol precipitation.

RESULTS

Mutagenesis of cysteine residues. Previous studies have shown that only oxidized OxyR is able to activate transcription (35). To determine whether the cysteine residues in OxyR were important for sensing the oxidative stress signal, we changed each of the six cysteines to serine by site-directed mutagenesis. The mutants were cloned into pUC18 and transformed into TA4112, an *E. coli* strain carrying a chromosomal deletion of *oxyR*. These strains were then assayed for their sensitivities to hydrogen peroxide and cumene hydroperoxide by using a growth inhibition assay. As seen in Table 2, the C25S, C143S, C180S, and C259S mutant strains showed the same sensitivity as a strain carrying the wild-type *oxyR* gene. The strains showed two distinct zones of inhibition for hydrogen peroxide; the first zone corresponded to complete killing, while the second zone corresponded to partial growth. The reason for this double zone is unclear, but the C208S mutant, although resistant, showed only one zone of inhibition for hydrogen peroxide. Unlike the C25S, C143S, C180S, C208S, and C259S mutants, the C199S mutant strain was as hypersensitive to hydrogen peroxide and cumene hydroperoxide as the control strain lacking *oxyR*, suggesting that the C199S mutant was unable to induce the expression of defense genes. The C199S protein was stable and able to specifically bind DNA (see below). Therefore, we propose that the C-199 residue is the possible redox center and that the C199S mutant is locked in the reduced conformation. A mutant in which all cysteine residues except C-199 were replaced by serine showed the same resistance as the C208S single mutant.

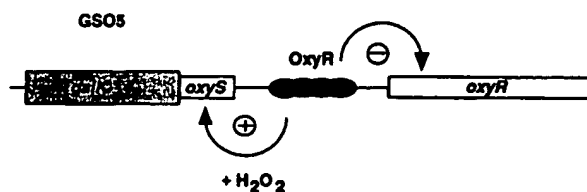


FIG. 1. *oxyS-galk* transcriptional fusion used to screen for OxyR mutants. When binding to the promoter region, OxyR represses its own expression, and upon oxidation, it activates *oxyS* transcription.

Screen for constitutively active OxyR mutants. Having identified C-199 as a critical amino acid for OxyR activity, we wanted to determine what other amino acids were required for OxyR to act as an activator. We chose to randomly mutagenize the entire *oxyR* gene and screen for OxyR mutants altered in their ability to activate transcription of the *oxyS* promoter. The *oxyS* gene encodes a small, untranslated regulatory RNA and is encoded divergently from the *oxyR* structural gene (4). The *oxyR* and *oxyS* promoters overlap, and OxyR bound to its site at these promoters acts to both repress *oxyR* expression (under oxidizing and reducing conditions) and activate *oxyS* expression (only under oxidizing conditions). We constructed a transcriptional fusion between the *oxyS* promoter and the *galk* reporter gene and integrated this fusion into the chromosome of *E. coli* SA2692 (Fig. 1). Subsequently, an *oxyR* deletion was moved into the strain by P1 transduction, giving rise to GSO5. Plasmids carrying *oxyR* were then mutagenized by hydroxylamine in vitro and introduced into the GSO5 background.

The abilities of the *oxyR* mutants to activate *oxyS* expression were monitored on MacConkey agar plates with galactose as a carbon source. For reasons that are not understood, wild-type OxyR displayed an uninducible or reduced phenotype on the MacConkey-galactose medium, even in the presence of hydrogen peroxide. The uninduced phenotype, however, suggested the possibility of screening for constitutively active mutants on this medium. We screened more than 10⁶ colonies for constitutively active mutants and identified 20 candidates. The mutants were tested for their sensitivities to hydrogen peroxide and cumene hydroperoxide in a growth inhibition assay, and 18 of the mutants showed smaller zones than the wild-type strain, indicating that they were more resistant to oxidants. Only the A233V mutant consistently had the two zones of growth inhibition seen for some of the cysteine mutants. Unlike the result shown in Table 2, only a single zone was observed for the strain expressing wild-type OxyR, probably because of differences in the strain backgrounds and vectors.

Mutations map to the C-terminal part of OxyR. To determine the locations and natures of the mutations, the mutant *oxyR* genes were sequenced entirely. Eight different mutations, all causing single amino acid exchanges, were found in the pool of 18 resistant candidates (Table 3). The A233T mutation, which caused a constitutive phenotype in our random screen, affected the same alanine residue mutated (A233V) in the original *oxyR2* constitutive mutant strain (14). Similarly, two different amino acid changes at position H-198 gave rise to a constitutive phenotype.

The positions of the mutations causing the constitutive phenotype are shown on an alignment of OxyR and five other members of the LysR family in Fig. 2. The location of the noninducible C199S mutation is also indicated in Fig. 2. All of the mutations are clustered in three regions in the C-terminal two-thirds of the protein, suggesting that these regions are

TABLE 3. Sensitivities of constitutively active *oxyR* mutant strains to oxidants

Strain	Codon exchange	Zone(s) of inhibition ^a (mm) with:		Phenotype ^b
		10% H ₂ O ₂	4% CHP	
Vector (pACYC184)		35	23	
Wild type		24	20	
C199S	TGT→TCT	42	30	Noninducible
T100I	ACA→ATA	24	12	Constitutive
H114Y	CAC→TAC	21	14	Constitutive
H198Y	CAC→TAC	21	13	Constitutive
H198R	CAC→CGC	23	14	Constitutive
R201C	CGC→TGC	23	16	Constitutive
C208Y	TGT→TAT	23	13	Constitutive
A233V ^c	GCA→GTA	21 (27)	13	Constitutive
A233T	GCA→ACA	16	12	Constitutive
G253K	GGG→AAG	19	13	Constitutive

^a Total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H₂O₂) or cumene hydroperoxide (CHP). The values are averages from two separate assays. The number in parentheses indicates the size of the second zone of partial growth.

^b Phenotype of mutants with respect to their color on MacConkey agar plates.

^c This mutant corresponds to the previously isolated *oxyR2* mutant (14).

involved in sensing oxidative stress and transducing the signal to RNA polymerase. One cluster of mutations affects residues T-100 and H-114, another cluster is evident around the critical cysteine C-199, and a third is located further downstream at

positions A-233 and G-253, which are at and near the original *oxyR2* mutation (A233V).

Mutants show constitutive *oxyS* transcription *in vivo*. The phenotype of the constitutive mutants on MacConkey agar plates suggested that, in contrast to the case for the wild-type protein, oxidation was not required for the constitutive mutants to activate *oxyS* transcription. To assess whether oxidation would increase the activities of the mutants and to compare the mutants with the wild-type protein *in vivo*, we divided cultures expressing the different *oxyR* mutants and treated half of each culture with hydrogen peroxide. Total RNA was then isolated from both the treated and untreated cells, and the levels of the *oxyS* message were determined by primer extension. Unlike the wild-type strain, all of the untreated constitutive mutants showed *oxyS* expression (Fig. 3). Only two of the mutants (T100I and H114Y) showed a two- to threefold induction of *oxyS* expression upon treatment with hydrogen peroxide. Although all of the constitutive mutants were active under reducing conditions, the overall level of activity varied from 3 to 400% of the wild-type activity. The noninducible C199S mutant did not show any activation of *oxyS*. The hydrogen peroxide-induced expression of two additional OxyR-regulated genes, *dps* and *kaiG*, was also tested for a subset of the mutants (H198Y, C199S, and A233V). All of the mutants showed the same relative expression seen with the *oxyS* gene, suggesting that the individual OxyR mutants had similar effects on all target genes (data not shown).

DNA binding by OxyR constitutive mutants. We assumed that OxyR binding to its target promoters is required for transcriptional activation. Previous studies had also shown that the oxidized and the reduced forms of OxyR have different DNA

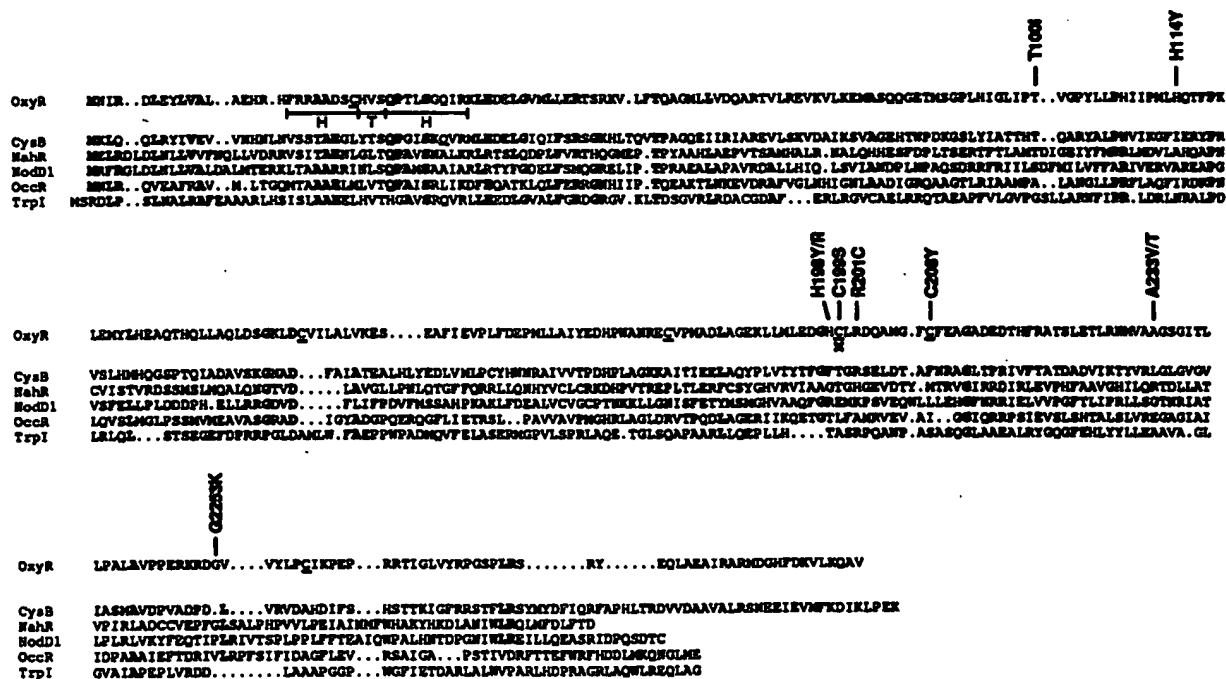


FIG. 2. Protein sequence alignment of six LysR family members and locations of the OxyR mutations causing a constitutively active phenotype. Residues that are identical in four of six sequences are in boldface, the bars mark the helix-turn-helix motif, and the six cysteine residues in OxyR are underlined. The x denotes the noninducible C199S mutant. The sequences were obtained from SWISS-PROT and GenBank (*E. coli* OxyR [OXYR_ECOLI], *E. coli* CysB [CYSB_ECOLI], *P. putida* NarR [NAHR_PSEPU], *Rhizobium meliloti* NodD1 [NOD1_RHIME], *P. aeruginosa* TrpI [TRPI_PSEAE], and *A. tumefaciens* OccR [TIPOCCR]). The alignment was done with the Genetics Computer Group program PILEUP with default parameters.

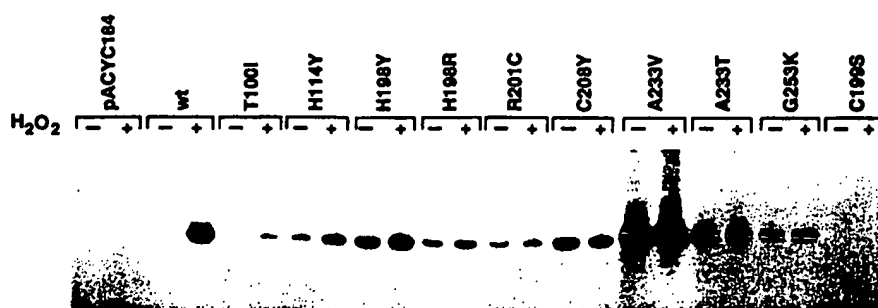


FIG. 3. Primer extension assays of *oxyS* induction in OxyR constitutive mutant strains. Total RNA was isolated from the corresponding *E. coli* strains, which were either untreated (–) or induced with 200 μ M hydrogen peroxide for 10 min (+). A labeled oligonucleotide capable of hybridizing to the *oxyS* transcript was then incubated with 3 μ g of total RNA and extended with reverse transcriptase. wt, wild type.

binding characteristics (35, 41). For example, at the *oxyR*-*oxyS* promoter, the reduced protein has a DNase I footprint that is extended compared with that of the oxidized protein. The reduced, but not the oxidized, footprint also shows a strong central DNase I-hypersensitive site. Therefore, to further characterize the mutants, we prepared cell extracts from Δ *oxyR*::*kan* strains expressing the wild-type and mutant OxyR proteins from pACYC184. We then assayed the extracts for binding to an *oxyR*-*oxyS* promoter fragment. If the mutations caused the proteins to be locked in the oxidized conformation, we should observe the shorter DNase I footprint characteristic of the oxidized wild-type protein. Alternatively, if the constitutive phenotype was caused only by the abnormal exposure of an activation domain, the mutants should bind in the extended conformation characteristic of reduced wild-type OxyR.

Since OxyR present in extracts prepared aerobically in the absence of high concentrations of dithiothreitol (DTT) is predominantly in the oxidized form (35), the lysate from wild-type cells gave rise to a short footprint (Fig. 4A). Among the constitutive mutants however, only G253K showed the shorter footprint. Four other constitutive mutants (H198Y, H198R, R201C, and C208Y) showed the extended DNase I footprint observed with the reduced wild-type protein. The extension was seen even when the extracts were treated with 0.2 or 2 mM hydrogen peroxide (data not shown). These results suggested that the H198Y, H198R, R201C, and C208Y proteins bind in the reduced configuration under oxidizing conditions. Reduced wild-type OxyR is not able to bind to the *katG* and *ahpC* promoter fragments, since these promoters lack determinants for reduced OxyR binding (41), and although H198R constitutively activates the *katG* and *ahpC* genes, we observed that the purified mutant protein did not give a footprint at either of these promoters (data not shown). However, the binding of RNA polymerase was found to alter the binding of H198R to the *oxyR*-*oxyS* fragment (see below), and an interaction with RNA polymerase may allow the H198R protein to bind to and constitutively activate the *katG* and *ahpC* promoters.

Unexpectedly, four constitutive mutants (T100I, H114Y, A233V, and A233T) behaved like E225K, a nonbinding mutant (described in reference 20), and did not show a distinct footprint even though equivalent amounts of OxyR protein were present in the samples (Fig. 4A and data not shown). Since the extracts for both the A233V and A233T mutants caused non-specific degradation of the *oxyR*-*oxyS* probe, we repeated the DNase I footprint experiment with purified A233V but still did not observe a footprint (see below). These binding studies demonstrated that T100I, H114Y, A233V, and A233T have decreased apparent affinities to DNA. We did detect a weak

shifted band for all four mutants in a more sensitive gel retardation assay, revealing some residual DNA binding activity which may be sufficient for activation of *oxyS* (data not shown). In addition, this weak binding must allow for autorepression by the mutant proteins, since none of the constitutively active mutants showed elevated levels of OxyR protein by immunoblot assays (data not shown). As shown below, the DNA binding affinities of some of the mutants may also be increased by the binding of RNA polymerase.

We also characterized the DNase I footprints of all the cysteine mutants created by site-directed mutagenesis (Fig. 4B). The C25S, C143S, C180S, and C259S mutants showed a short footprint, while the C199S and C208S mutants gave an extended footprint with the hypersensitive cleavage site seen with the reduced wild-type protein. The C208S mutant also showed a slightly decreased binding to the DNA compared with the wild-type protein and the other C-to-S mutants.

Transcriptional activation by mutant OxyR proteins *in vitro*. To study some of the different OxyR mutants *in vitro*, we overexpressed and purified the noninducible C199S protein and two representative constitutive mutant proteins, H198R and A233V (Fig. 5). We also overexpressed and purified two nonbinding mutants, S33N and E225K (described in the accompanying paper [20]). We then assayed the abilities of the purified wild-type and mutant proteins to activate transcription *in vitro*. Aliquots of the purified proteins were incubated with a supercoiled plasmid carrying the *oxyS* and *bla* (conferring ampicillin resistance) genes together with purified RNA polymerase holoenzyme in a transcription reaction. Subsequently, the *in vitro*-synthesized *oxyS* and *bla* transcripts were detected by primer extension assays (Fig. 6). A distinct OxyR-dependent signal was observed for *oxyS* with the wild-type protein (Fig. 6A), since aerobically purified wild-type OxyR is predominantly oxidized (35). We did not detect an *oxyS* transcript with C199S, consistent with our observations that this mutant was unable to activate transcription *in vivo*. The constitutive H198R mutant was nearly as active as the wild-type protein, but the constitutive A233V protein showed significantly less activity than either wild-type OxyR or H198R. The low level of activity of the A233V mutant *in vitro* was surprising, since this mutant is the strongest activator *in vivo*. Possibly the A233V protein is altered during purification or requires additional factors for stabilization (discussed in reference 20). The non-binding mutant E225K showed very weak activity, while the second nonbinding mutant, S33N, did not show any detectable activity. The expression of the control *bla* gene was unaffected by the different OxyR mutants.

To compare the activities of the wild-type protein and the

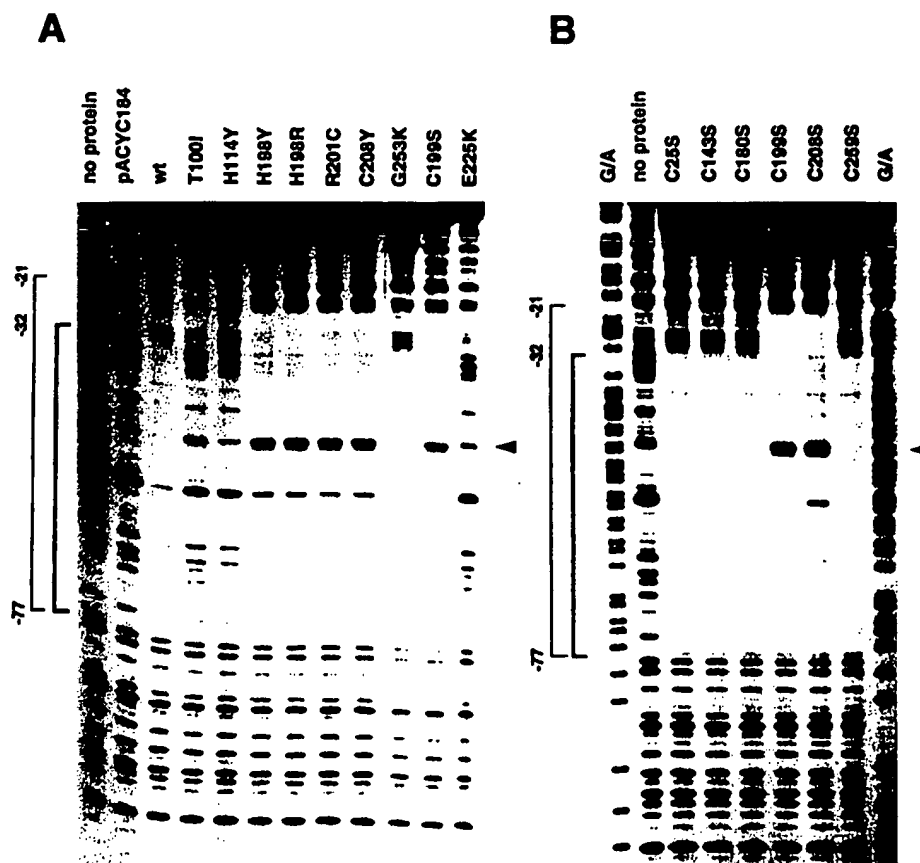


FIG. 4. DNase I footprints of the OxyR mutants binding to the top strand of the *oxyS* promoter. The 100-bp *EcoRI-HindIII* fragment of pGSO40 (41) labeled at the *HindIII* site was incubated with 2 μ l of the soluble fraction of crude cell extracts. (A) Constitutive *oxyR* mutations carried on pACYC184; (B) cysteine mutations carried on pUC18. The heavy bracket indicates the oxidized shorter footprint of OxyR, the light bracket indicates the reduced extended footprint of OxyR, and the arrowhead indicates the hypersensitive cleavage site seen with the extended footprint. The positions of the footprints are labeled with respect to the start of the *oxyS* transcript, and the G/A sequence of the fragment is shown in panel B. wt, wild type.

constitutive mutant H198R protein under reducing conditions, the *in vitro* transcription assays were also carried out in presence of 200 mM DTT (Fig. 6B). For the wild-type protein, the activity under reducing conditions dropped to 12% of the activity in the absence of DTT, while for the H198R mutant, the activity was only reduced to 48% of the activity in the absence of DTT, showing that the H198R protein is less sensitive to reducing conditions. These observations agree with our findings that H198R is active under normal, reducing conditions *in vivo*.

Cooperative binding between mutant OxyR proteins and RNA polymerase. Tao and colleagues have shown that OxyR has a cooperative effect on RNA polymerase binding to the *katG* promoter site (36). To test whether the mutant proteins affected polymerase binding, we analyzed the DNase I footprint of RNA polymerase binding to the *oxyR-oxyS* promoter fragment in the presence of the purified OxyR mutants (Fig. 7A). RNA polymerase (0.3 to 10 pmol) alone did not show a footprint at the *oxyS* promoter (Fig. 7A, lane 2). However, when the polymerase was incubated with oxidized wild-type OxyR, a clear protection due to polymerase was observed at the *oxyS* promoter (Fig. 7A, lane 4). The uninducible mutant, C199S, showed an extended footprint alone (Fig. 7A, lane 5)

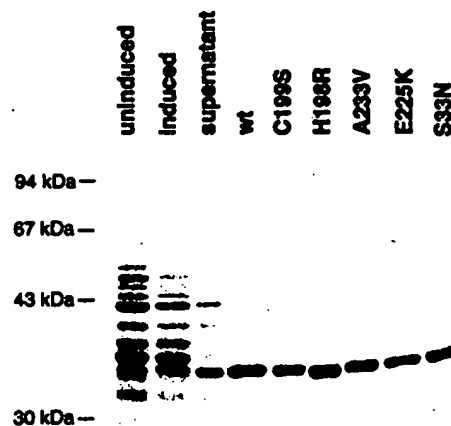


FIG. 5. Overexpression and purification of OxyR mutant proteins. Aliquots of uninduced and induced cultures, the soluble fraction of induced cells (described in Materials and Methods), and purified protein fractions (80 to 100 μ g) were electrophoresed on an SDS-12% PAGE gel and stained with Coomassie blue. The A233V and E225K proteins showed reduced affinities to the heparin and eluted at lower salt concentrations than the wild-type protein, which likely accounts for the contaminating protein band in these samples. The mobilities of the standard proteins are indicated to the left of the gel. wt, wild type.

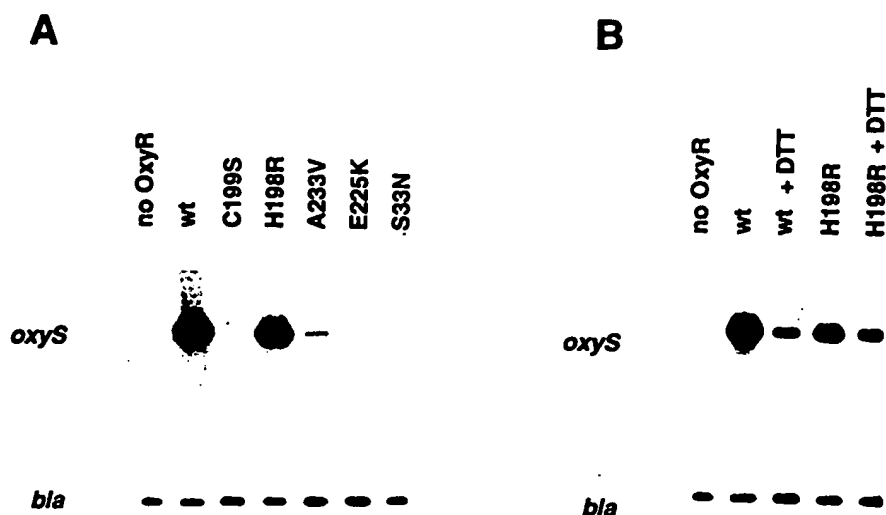


FIG. 6. In vitro transcription assays of purified wild-type (wt) and OxyR mutant proteins. The transcription reactions were carried out as described in Materials and Methods. The transcripts were then detected with *oxyS*- and *bla*-specific oligonucleotides in a primer extension assay. (A) Assays with the purified wild-type and C199S, H198R, A233V, E225K, and S33N mutant proteins under oxidizing conditions (no DTT); (B) assays with the wild-type and constitutive H198R mutant proteins in the absence or presence of 200 mM DTT.

and did not induce RNA polymerase binding (lane 6), consistent with the lack of transcription activation seen with this mutant. In contrast, the constitutive H198R mutant, which also showed an extended footprint alone (Fig. 7A, lane 7), had a cooperative effect on polymerase binding (lane 8). Interestingly, the strong DNase I-hypersensitive site detected for H198R alone was not seen in the presence of polymerase. The A233V constitutive mutant, which did not bind to the DNA alone (Fig. 7A, lane 9), nevertheless stimulated RNA polymerase binding (lane 10), although the binding was weaker than that observed with H198R. The nonrepressing mutants E225K and S33N (described in the accompanying paper [20]) also did not bind to DNA alone (Fig. 7A, lanes 11 and 13), but E225K could still induce some cooperative binding of RNA polymerase (lane 12). These footprints showed that the constitutive mutants (H198R and A233V), but not the noninducible mutant (C199S), had a cooperative effect on RNA polymerase binding to the *oxyS* promoter. The studies also showed that an interaction with RNA polymerase increased the DNA binding affinity of the A233V and E225K mutant proteins.

We examined RNA polymerase binding with wild-type OxyR and H198R in the presence of 200 mM DTT (Fig. 7B). The wild-type protein did not increase the binding of polymerase under the reducing conditions (Fig. 7B, lane 7), while the constitutively active H198R mutant still had a cooperative effect on polymerase in the presence of the 200 mM DTT (lane 11). These observations support the conclusion that H198R is constitutively active and that the ability to induce RNA polymerase-promoter binding correlates with the ability to activate transcription.

DISCUSSION

In this study, we used site-directed and random mutageneses to isolate one noninducible and eight constitutively active OxyR mutants. Together with the nonbinding mutants de-

scribed in the accompanying paper (20), these mutants have allowed us to define functional domains in the OxyR protein.

C-199 as a possible redox center. Using site-directed mutagenesis, we found that only one (C-199) of six cysteine residues was critical for the induction of defense genes by OxyR. The C199S mutant was unable to activate the antioxidant genes and gave rise to an extended footprint characteristic of reduced OxyR. Since oxidation is necessary for OxyR to activate transcription in vitro, we suggest that C-199 is the redox center of the protein and that the C199S protein is locked in the reduced conformation. Cysteines are known to be redox-reactive amino acids and can form inter- or intramolecular disulfide bridges. However, an intramolecular disulfide bridge is unlikely to be the redox-active center of OxyR, since only C-199 proved to be critical, and the mutant in which all other cysteines were mutated to serine was still active in vivo. We did not observe any differences between the wild-type and mutant C199S proteins on nonreducing gels which permit the resolution of disulfide-linked oligomers (data not shown), suggesting that intermolecular disulfide bridges do not constitute the redox-active center. Redox-active metals are also coordinated by cysteine residues. The SoxR protein, a transcriptional activator in the bacterial response to superoxide, carries a nonheme iron which is likely to be the redox-reactive center of the SoxR protein (17). However, we do not think that a metal coordinated through C-199 is the redox-active center of OxyR, since the addition of chelators did not affect transcriptional activation of OxyR in vitro (35). Cysteines have been shown to be reversibly oxidized to sulfenic (SO_2^-) or sulfinic (SO_2^-) acid and irreversibly oxidized to sulfonic (SO_3^-) acid. Both a streptococcal NADH peroxidase and an oxidation product of the protease papain have been reported to have a cysteine-sulfenic acid (22, 27). Since OxyR is reversibly oxidized and only a single cysteine is critical for OxyR activity, we suggest that C-199 is oxidized to a sulfenic acid. The mutants described

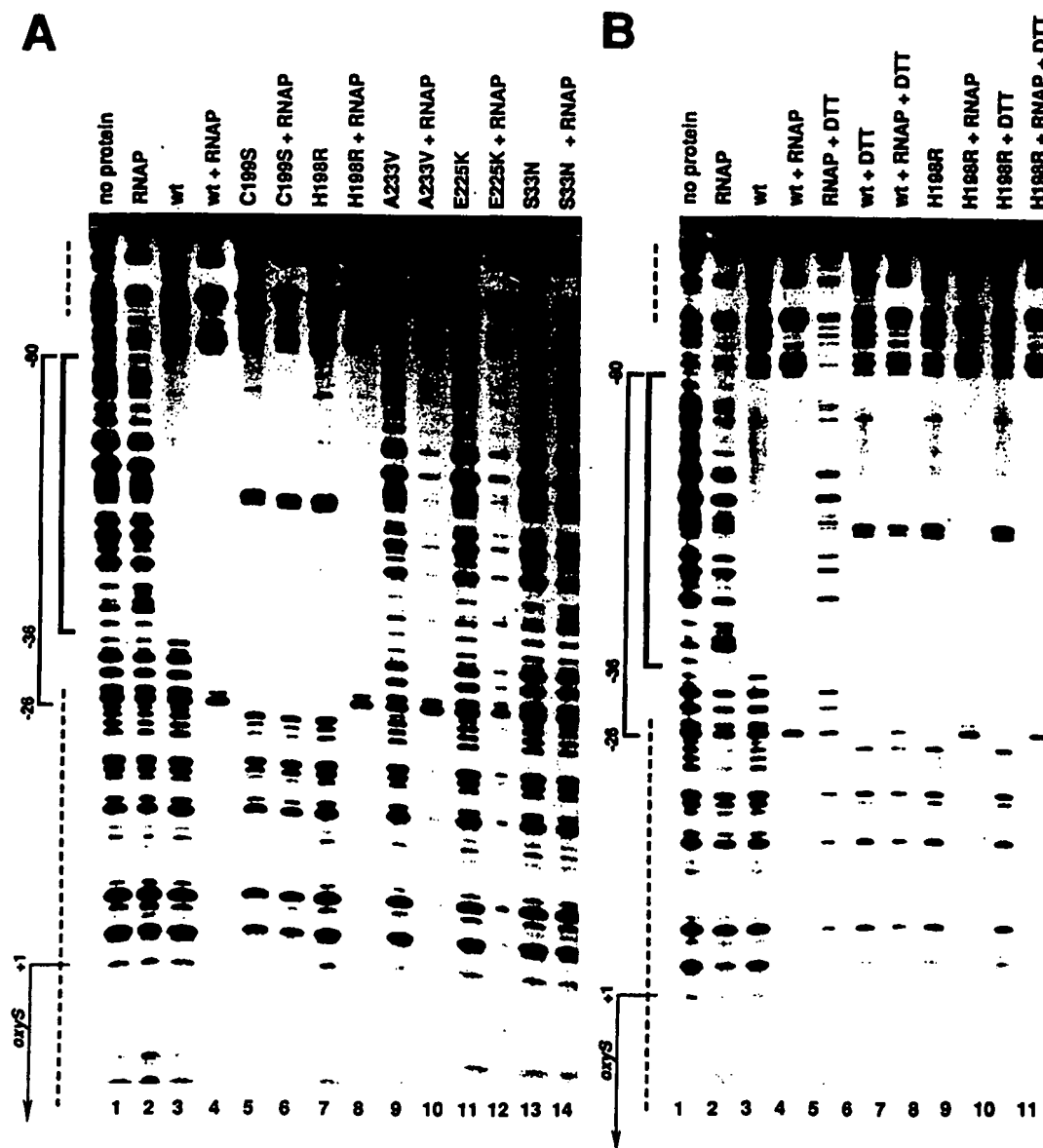


FIG. 7. DNase I footprints of RNA polymerase (RNAP) together with wild-type (wt) and mutant OxyR proteins on the bottom strand of the *oxyS* promoter fragment. The 200-bp *EcoRI-HindIII* fragment of pGSO43 (41) labeled at the *EcoRI* site was incubated with purified RNA polymerase and the purified wild-type and OxyR mutant proteins as indicated. (A) Footprinting assays carried out in the absence of DTT; (B) RNA polymerase binding assayed in the absence or presence of 200 mM DTT. The heavy brackets denote the oxidized footprint of OxyR, the light brackets denote the reduced extended footprint of OxyR, and the dotted lines indicate the sequences protected by RNA polymerase binding. The positions of the footprints are labeled with respect to the start of the *oxyS* transcript. The upper protection by RNA polymerase does not correspond to either the *oxyS* or *oxyR* promoter and is assumed to be due to nonspecific binding to the end of the fragment.

here will be useful for future biochemical studies of the modifications of the cysteine residues in OxyR.

Constitutively active mutants define inducer-responsive and activation domains. Using a random mutagenesis approach, we isolated eight mutants which constitutively activate transcription of their target genes during normal growth and show increased resistance to oxidants. The corresponding mutations can be grouped and map to domains that may be functionally conserved among the LysR family members. Two of the amino acid substitutions (T100I and H114Y) map to a region (resi-

dues 98 to 150) that is somewhat conserved among the LysR proteins and that has been defined as a coinducer recognition/response domain by mutations in other LysR family members (29). Four of the mutations (H198Y, H198R, R201C, and C208Y) map within or near a second coinducer recognition/response domain (residues 196 to 206) of the LysR family (29). These four mutations are located close to the proposed redox-active C-199 residue, consistent with the conclusion that this region is the hydrogen peroxide-responsive domain. Finally, three of the mutations (A233V, A233T, and G253K) are lo-

cated near a carboxy-terminal region (residues 227 to 253) that shows some homology between the LysR proteins. Given the partial conservation of residues 227 to 253 and residues 98 to 150, these regions might be involved in touching RNA polymerase. Experiments described in the accompanying paper also indicate that the C-terminal domain is involved in oligomerization of OxyR (20).

It is not clear whether the critical C-199 residue is still modified by oxidation in the constitutive mutants. However, since the T100I and H114Y mutants still showed a two- to threefold increase of activation upon oxidation, these mutants should still be redox reactive. Three of the mutations causing a constitutively active phenotype affected histidine residues (H114Y, H198Y, and H198R). The crystal structures of the streptococcal NADH peroxidase and the oxidized papain (the two proteins found to have a sulfenic acid) showed that histidine residues form hydrogen bonds with the redox-active cysteine (19, 32). If a sulfenic acid proves to be the redox center of OxyR, the H-114 and H-198 residues may act to form hydrogen bonds with the sulfenic acid. We suggest that the amino acids of the constitutive mutants may more effectively stabilize the negatively charged residue and allow the oxidation of C-199 during normal growth. Structural studies of OxyR should help to elucidate whether the regulator carries a sulfenic acid stabilized by histidine residues and give additional insights into the domains of OxyR and other LysR family proteins.

Cooperative binding with RNA polymerase. The overall activities of the constitutive mutants varied from 3 to 400% of the wild-type activity *in vivo*. It was not possible to correlate the variations in activity with differences in DNA binding. In fact, we were surprised to find that although one constitutive mutant gave rise to a short oxidized footprint, several constitutive mutants showed reduced extended footprints, and some of the mutants showed no footprint at all. The different footprint phenotypes were specific to different regions, since both T100I and H114Y had decreased binding and all of the mutants with substitutions near C-199, including the C208S mutant generated by site-directed mutagenesis, gave extended footprints. The ability to activate transcription, however, did correlate with the abilities of the purified proteins to bind cooperatively with RNA polymerase. The uninducible C199S mutant did not induce transcription and was unable to stimulate RNA polymerase binding. In contrast, the constitutive H198R mutant, which showed the same extended reduced footprint seen for C199S in the absence of RNA polymerase, induced polymerase binding even in the presence of 200 mM DTT. Another constitutive mutant, A233V, showed significantly reduced binding to DNA alone but also induced polymerase binding.

RNA polymerase binding to the promoter can be a key step in the activation by transcription factors. At some bacterial promoters, RNA polymerase is unable to bind in the absence of a transcriptional regulator, preventing the titration to these promoters when the corresponding genes need not be activated. For example, RNA polymerase is unable to bind to the promoters of the *pho* (phosphate) genes in the absence of the PhoB activator (23). We propose that the oxidation of the wild-type OxyR protein exposes a domain which allows OxyR to recruit RNA polymerase and that the constitutive phenotype of our mutants may be due to exposure of this domain under both oxidizing and reducing conditions.

Our results also suggest that while OxyR induces RNA polymerase binding, RNA polymerase has an effect on OxyR. Both A233V and E225K did not show DNase I footprints when incubated with the *oxyS* promoter alone but did bind in the presence of RNA polymerase. We propose that the contact

with polymerase may also cause the H198R mutant to adopt the oxidized configuration, since the DNase I-hypersensitive site in the center of the reduced H198R footprint disappears upon the interaction with RNA polymerase. An important direction for further studies will be to elucidate this reciprocal interaction between OxyR and RNA polymerase.

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Mutational Analysis of the Redox-Sensitive Transcriptional Regulator OxyR: Regions Important for DNA Binding and Multimerization

INES KULLIK,[†] JENNIFER STEVENS,[‡] MICHEL B. TOLEDANO,[§] AND GISELA STORZ*

Cell Biology and Metabolism Branch, National Institute of Child Health and
Human Development, Bethesda, Maryland 20892

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OxyR is a LysR-type transcriptional regulator which negatively regulates its own expression and positively regulates the expression of proteins important for the defense against hydrogen peroxide in *Escherichia coli* and *Salmonella typhimurium*. Using random mutagenesis, we isolated six nonrepressing OxyR mutants that were impaired in DNA binding. Five of the mutations causing the DNA binding defect mapped near the N-terminal helix-turn-helix motif conserved among the LysR family members, confirming that this region is a DNA binding domain in OxyR. The sixth nonrepressing mutant (with E-225 changed to K [E225K]) was found to be predominantly dimeric, in contrast to the tetrameric wild-type protein, suggesting that a C-terminal region defined by the E225K mutation is involved in multimerization.

The *Escherichia coli* OxyR protein is a redox-sensitive transcriptional regulator which activates the expression of antioxidant defense genes under oxidizing conditions. During normal growth and upon oxidative stress, OxyR also acts as a repressor and negatively autoregulates its own expression and the expression of the Mu phage *mom* gene (8, 16, 34). OxyR specifically binds upstream of the promoters it regulates, but the seven natural binding sites which have been characterized only show limited homology (35). Recent studies of 54 synthetic binding sites, however, allowed the definition of an oxidized-OxyR binding motif composed of four ATAGxt elements (37). OxyR-DNase I footprints are long and cover 45 bp, and hydroxyl radical footprinting and interference assays showed that the oxidized OxyR protein binds to the four ATAGxt elements by contacting the DNA in four adjacent major grooves (37). These footprinting assays also showed that OxyR binding is different under oxidizing and reducing conditions (32, 37).

OxyR is a member of the family of LysR-type transcriptional regulators (8, 16, 33, 39). LysR family members are DNA-binding proteins which positively regulate expression of their target genes and often also negatively regulate their own expression (reviewed in reference 28). Sequence comparisons among LysR family members have shown that the region encompassing the 66 N-terminal amino acids exhibits the greatest sequence identity and includes a helix-turn-helix (HTH) motif likely to be a DNA binding domain (28). Mutations which map to the HTH region lead to a loss of DNA binding by *Pseudomonas putida* NahR (29), *Rhizobium leguminosarum* NodD (11), and other LysR-type proteins. Parts of the C-terminal domains of LysR-type proteins also seem to contribute to DNA binding, since several mutations in this region of *P. putida* NahR (29) and *Citrobacter freundii* AmpR (6) affect DNA binding.

Like OxyR, other LysR family members protect unusually long regions from DNase I digestion. The long binding sites suggest that the LysR proteins may be multimeric, and *E. coli* MetR (25), *Rhizobium meliloti* NodD3 (17), and *Klebsiella aerogenes* Nac (19) have been reported to be dimers, while NahR (29), *Pseudomonas aeruginosa* TrpI (14), and *Salmonella typhimurium* CysB (26) have been found to be tetramers. The regions involved in multimerization are not yet well defined; however, some of the C-terminal mutations in NahR and also in AmpR are not negative *trans* dominant, suggesting that they might affect a multimerization domain (6, 29).

In this study, we used random mutagenesis to define regions of the OxyR protein involved in DNA binding. We were able to show that the proposed HTH motif in OxyR is a DNA binding domain, since several mutations in this region led to impaired DNA binding of the mutant proteins. Moreover, we found that the oxidized and reduced forms of OxyR are predominantly tetrameric, while one binding mutant and one constitutively active mutant, described in the accompanying paper (21), were dimeric, suggesting that the C-terminal region affected by these mutations is involved in tetramerization.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are described in Table 1. The strain GSO7 was constructed as follows. The 0.2-kb *HindIII*-*SmaI* fragment of pAQ17 carrying the *oxyS* and *oxyR* promoter region (16) was cloned into the unique *SmaI* site of pTS7 (1) to create the *galK* fusion. The plasmid was then recombined onto λ -*g2055* (c1857 *galK* *nin5*) and integrated into the *att* site of SA2692. A Δ *oxyR::kan* deletion-insertion mutation was subsequently moved into the strain by P1 transduction (3). The pACYC184 derivative pAQ5 used for the mutagenesis contains *oxyR* on a *Bam*HI-*Eco*RV fragment (31). A *Bam*HI-*Hind*III fragment of these clones was moved into M13mp18 for sequencing. All sequencing and subcloning were carried out by standard procedures.

Media and growth conditions. Strains were grown in LB medium (27), and ampicillin (100 μ g/ml [final concentration]), kanamycin (25 μ g/ml), chloramphenicol (25 μ g/ml), or tetracycline (15 μ g/ml) was added when appropriate. The resistance of strains to hydrogen peroxide and cumene hydroperoxide was assayed by zones of inhibition, which were determined as described previously (15) except that the strains were grown in and plated on LB medium containing the appropriate antibiotics.

Mutagenesis. Approximately 10 μ g of purified pAQ5 plasmid DNA was randomly mutagenized with hydroxylamine as described in the accompanying paper (21). The mutagenized DNA mixture (5 to 10 μ l) was then used to transform *E. coli* XL1-blue cells directly. The transformants were rinsed off the plates, and the plasmid DNA was isolated and used to transform the GSO7 recipient strain.

* Corresponding author. Mailing address: National Institutes of Health, Building 18T, Room 101, Bethesda MD 20892-5430. Phone: (301) 402-0968. Fax: (301) 402-0078. Electronic mail address: storz@helix.nih.gov.

[†] Present address: Institut für Medizinische Mikrobiologie, Universität Zürich, CH-8028 Zürich, Switzerland.

[‡] Present address: University of Virginia, Charlottesville, VA 22903.

[§] Present address: Department of Pharmacology and Toxicology, Rutgers University College of Pharmacy, Piscataway, NJ 08855-1179.

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
XL1-blue	F' [<i>proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ <i>M15</i> Tn10 (Tc ^r)]	10
D1210	HB101/F' [<i>lacI</i> ^q]	7
TA4484	<i>oxyR</i> Δ3, pMC7	36
SA2692	HB101 <i>recA</i> ⁺ Δ <i>lac</i> Δ <i>gal</i> -165	1
GSO7	SA2692 Δ <i>oxyR::kan</i> (λ _{Y2055} <i>oxyR-galk</i>)	This study
GSO27	SA2692 (λ _{Y2055} <i>oxyR-galk</i>)	This study
Plasmids		
pTS7	pBR322 <i>int</i> P ⁺ P; promoterless <i>lacZ</i> and <i>galETK</i> , Ap ^r	1
pMC7	<i>lacI</i> ^q Tc ^r	12
pACYC184	Cm ^r Tc ^r	13
pKK177-3	P _{lac} promoter, derivative of pKK223-3, Ap ^r	9
pAQ5	<i>oxyR</i> wt ^a in pACYC184, Cm ^r	31
pAQ25	<i>oxyR</i> wt in pKK177-3 with altered SD ^b sequence, Ap ^r	32
pGSO61	<i>oxyR</i> R4C in pACYC184	This study
pGSO62	<i>oxyR</i> T31M in pACYC184	This study
pGSO63	<i>oxyR</i> L32F in pACYC184	This study
pGSO64	<i>oxyR</i> S33N in pACYC184	This study
pGSO65	<i>oxyR</i> R50W in pACYC184	This study
pGSO66	<i>oxyR</i> E225K in pACYC184	This study
pGSO68	<i>oxyR</i> C199S in pKK177-3	21
pGSO69	<i>oxyR</i> A233V in pKK177-3	21
pGSO70	<i>oxyR</i> E225K in pKK177-3	21

^a wt, wild type.^b SD, Shine-Dalgarno.

Finally, the GSO7 transformants were screened for the desired phenotype on MacConkey agar plates.

Primer extension assays. Cells were grown to an optical density at 600 nm of 0.4, and then half of each sample was treated with hydrogen peroxide (200 μM final concentration) for 10 min. Total RNA was isolated by using hot phenol, and 0.1 pmol of an end-labeled *oxyS* oligonucleotide (5'-GCAAAAGTTCACGT TGG) was annealed to 3 μg of total RNA as described previously (30 [short protocol]). The extension reaction was performed with Superscript reverse transcriptase from Gibco BRL (Gaithersburg, Md.) in the reaction buffer provided. The extension products were separated on an 8% sequencing gel and were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Immunoblot assays. Proteins were separated on a sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis gel (22) and transferred to a nitrocellulose filter by electroblotting. The filter was then probed with a 1:10,000 dilution of antibodies to an *OxyR*-β-galactosidase fusion protein (32). Bound antibody was visualized with rabbit antiserum by using the enhanced chemiluminescence Western blotting (immunoblotting) system from Amersham, Arlington Heights, Ill.

DNA binding assays. Binding reactions were carried out as described previously with 5,000 cpm of an end-labeled DNA fragment and either 1 to 10 ng of pure protein (21) or 1 to 2 μl of the soluble fraction of a crude cell lysate (36). For the mobility shift assays, the binding reaction mixtures were loaded on a nondenaturing, low-ionic-strength, 8% polyacrylamide gel (5). To prepare the crude cell extract, the pellet from a 5-ml overnight culture was sonicated in 800 μl of 10 mM Tris buffer (pH 8) containing 20% glycerol. The insoluble fraction was then removed by centrifugation.

Galactokinase assays. The samples for the galactokinase assays were obtained as described previously (2). The assays were carried out as described by Wilson and Hogness (40). D-[1-¹⁴C]galactose (55 mCi/mmol; 200 Ci/ml) was obtained from Amersham, and the ion-exchange filter paper (DE81) was obtained from Whatman (Maidstone, England).

Gel chromatography. About 1 to 2 mg of pure protein (21) mixed with standard proteins (Bio-Rad, Richmond, Calif.) in a 200-μl volume was applied to a Superose 12 gel filtration column (Pharmacia, Piscataway, N.J.) equilibrated with buffer Z (21) containing 0.3 M KCl. The proteins were eluted with the same buffer Z, and aliquots of the column fractions were analyzed by immunoblots.

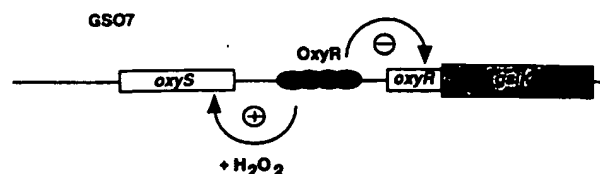


FIG. 1. *oxyR-galK* transcriptional fusion used to screen for *OxyR* nonrepressing mutants. *OxyR* bound to the *oxyR-oxyS* promoter region represses its own expression under both oxidizing and reducing conditions and activates *oxyS* transcription upon oxidation.

RESULTS

Screen for nonrepressing *OxyR* mutants. To characterize *OxyR* domains required for DNA binding, we chose to randomly mutagenize the entire *oxyR* gene and screen for mutants defective in DNA binding. To easily identify mutants, we constructed a transcriptional fusion between the *oxyR* promoter and the *galK* reporter gene. When *OxyR* binds to this site at the overlapping *oxyR* and *oxyS* promoters (Fig. 1), *OxyR* represses the expression of the *oxyR* gene independent of the oxidative state of the protein. Under oxidizing conditions, *OxyR* also acts to induce expression of the divergently transcribed *oxyS* gene, which encodes a small, untranslated regulatory RNA (4). Since binding of the *OxyR* protein to the DNA is assumed to be required for repression, mutant *OxyR* proteins which are not able to repress *oxyR* expression are likely to be DNA-binding mutants. The *oxyR-galK* fusion was integrated into the chromosome of *E. coli* SA2692, and subsequently a Δ*oxyR::kan* deletion was moved into the strain by P1 transduction to generate GSO7. Plasmids carrying the *oxyR* gene were mutagenized by hydroxylamine in vitro and introduced into this background. The abilities of the different mutants to repress *oxyR* expression were monitored on MacConkey agar plates containing galactose as a carbon source. In GSO7, wild-type *OxyR* repressed the expression of the *oxyR-galK* fusion, resulting in white colonies, while mutants that were unable to repress this fusion were detected as red colonies.

More than 10⁶ colonies were screened for nonrepressing mutants with the *oxyR-galK* fusion strain, and 32 candidates were isolated. Since a truncated or unstable form of *OxyR* would also lead to red colonies in GSO7, we checked whether crude extracts of strains harboring the corresponding *oxyR* mutations expressed a full-length *OxyR* protein. Ten of the 32 candidates did not show any detectable *OxyR* protein in an immunoblot assay, and one of the candidates expressed a truncated protein. The other 21 candidates showed a full-length protein, and the expression of *OxyR* was elevated approximately 50-fold compared with that in the wild-type strain (see below), consistent with the observation that these mutants are unable to act as repressors of the *oxyR* promoter.

The mutants were then tested for their sensitivities to hydrogen peroxide and cumene hydroperoxide in a growth inhibition assay. All showed a larger killing zone than the wild-type strain, indicating that they were more sensitive to the oxidants than the wild-type strain (Table 2). The increased sensitivity to oxidants, and also to peroxides presumed to be in the medium, most likely accounts for the impaired ability of the mutant strains to form single colonies. As observed for the Δ*oxyR::kan* strain, the single colonies grew only near heavy streaks of cells.

Five nonrepressing mutations map near the HTH motif. To determine the locations and natures of the mutations, the mutant *oxyR* genes were sequenced entirely. Six different mutations, all causing single amino acid changes, were found

TABLE 2. Sensitivities of nonrepressing *oxyR* mutant strains to oxidants

Strain ^a	Codon exchange	Zone of inhibition ^b (mm) with:	
		10% H ₂ O ₂	4% CHP
Vector (pACYC184)		35	23
Wild type		24	20
R4C	CGT→TGT	41	34
T31M	ACG→ATG	41	26
L32F	CTT→TTT	33	25
S33N	AGC→AAC	43	33
R50W	CGG→TGG	32	21
E225K	GAA→AAA	26	22

^a All mutants had a nonrepressing phenotype with respect to their color on MacConkey agar plates.

^b Total diameter of the zone of inhibition caused by the addition of hydrogen peroxide (H₂O₂) and cumene hydroperoxide (CHP). The values are averages from two separate assays.

among the 21 nonrepressing candidates (Table 2). With one exception, all of the nonrepressing mutations were located in or within 15 amino acids of the HTH motif of the OxyR protein presumed to be involved in DNA binding (Fig. 2). The region around the HTH motif displays the highest level of sequence similarity among LysR-type proteins, and amino acid S-33 is one of the most conserved amino acids. We found that the mutant with a change of S-33 to N (S33N) had a very severe mutant phenotype and was even more sensitive to hydrogen peroxide and cumene hydroperoxide than the $\Delta oxyR::kan$ strain. The mutations R4C and R50W also affect strongly con-

served amino acids. One mutation, E225K, is not located near the HTH motif, and the failure of this mutant to repress *oxyR* expression is thought to be due to a defect in multimerization of the protein (see below).

One nonrepressing mutant protein was sequenced even though immunoblot analysis revealed the protein to be slightly smaller than the wild-type OxyR protein. This mutant carried an amber mutation at position 283 and was not able to complement an *oxyR* deletion strain (data not shown). Since a C-terminal deletion of only 22 amino acids resulted in a non-repressing phenotype, this region may be important for DNA binding and may contact the DNA, be required for multimerization, or be crucial for the appropriate folding of the protein. A previously described strain expressing OxyR with a deletion of only 11 C-terminal amino acids showed a wild-type phenotype in the peroxide sensitivity assay, suggesting that this truncated protein is still able to bind DNA (data not shown) (16).

Nonrepressing mutants show decreased expression of defense genes. The ability of OxyR to bind DNA was assumed to be required for both repression and activation of transcription. Since all of the nonrepressing mutants exhibited increased sensitivity to oxidants, we examined whether the mutant proteins were impaired in activating transcription of defense genes in vivo and whether the activity of the mutants would still be affected by oxidation. Total RNA was isolated from untreated and hydrogen peroxide-treated cultures of strains expressing the different nonrepressing mutants. The levels of the OxyR-activated *oxyS* message were then determined by primer extension assays (Fig. 3). As expected, some of the mutants (T31M, S33N, and R50W) showed no activation of *oxyS*. Three mutants (R4C, L32F, and E225K), however, could still activate transcription under oxidizing conditions, although the abilities

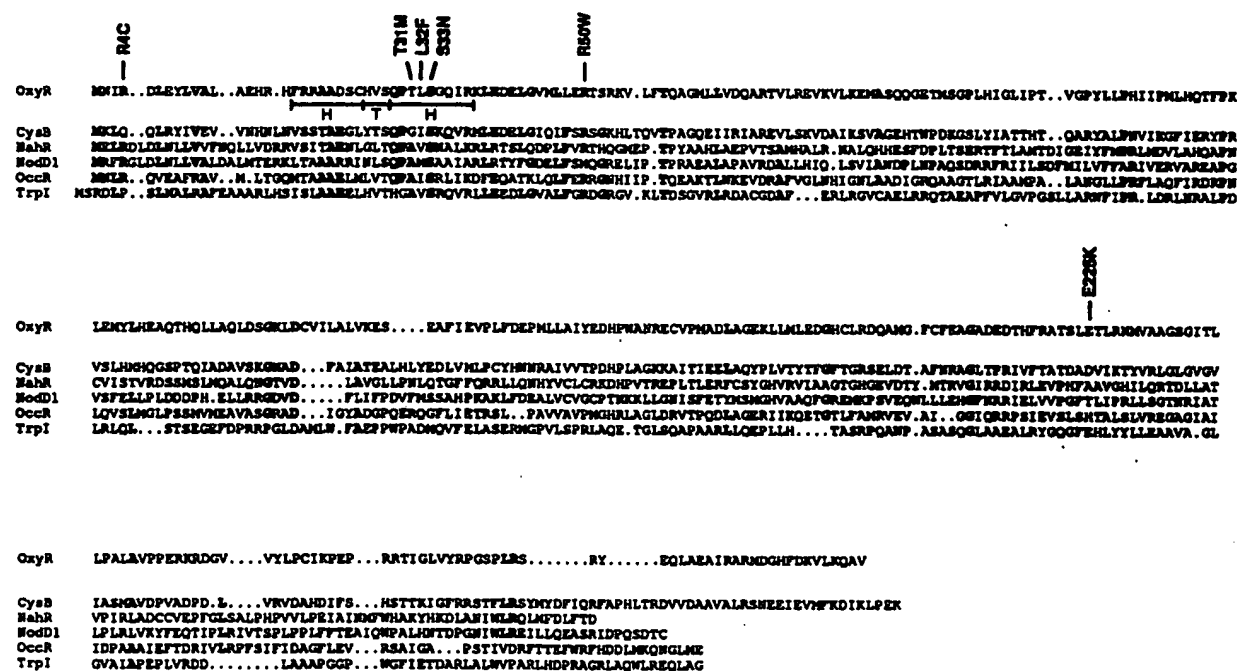


FIG. 2. Protein sequence alignment of six LysR family members and locations of the OxyR mutations causing a nonrepressing phenotype. Residues that are identical in four of six sequences are in bold-face, and the bars mark the region of the HTH motif. The sequences were obtained from SWISS-PROT and GenBank (*E. coli* OxyR [OXYR_ECOLI], *E. coli* CysB [CYSB_ECOLI], *P. putida* NahR [NAHR_PSEPU], *R. melioli* NodD1 [NOD1_RHIME], *P. aeruginosa* TrpI [TRPI_PSEAE], and *Agrobacterium tumefaciens* OccR [TPOCCR]). The alignment was done with the Genetics Computer Group program PILEUP with default parameters.

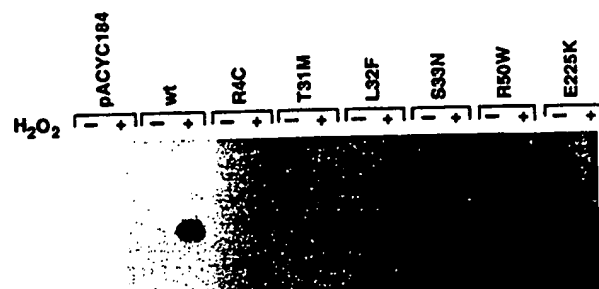


FIG. 3. Primer extension assays of *oxyS* induction in nonrepressing OxyR mutants. Exponential-phase cultures expressing the indicated OxyR mutants were split, and half of each culture was treated with 200 μ M hydrogen peroxide for 10 min. Total RNA was then isolated from the treated (+) and untreated (-) cells, and a labeled oligonucleotide capable of hybridizing to the *oxyS* transcript was incubated with 3 μ g of each RNA sample and extended with reverse transcriptase. wt, wild type.

of these mutants to induce *oxyS* expression were reduced to 5 to 15% of the wild-type activity. The S33N mutant was also assayed for its effect on the expression of the OxyR-regulated *ahpC* and *dps* genes, but as with *oxyS*, no transcriptional activation was detected (data not shown).

DNA binding by nonrepressing mutants. We next examined the DNA binding properties of the nonrepressing mutants by DNase I footprinting and DNA mobility shift assays. Extracts from strains carrying a chromosomal deletion of *oxyR* and either the wild-type or the mutant *oxyR* genes on pACYC184 were assayed for binding to an *oxyR-oxoS* promoter fragment. No DNase I footprint was observed for the nonrepressing

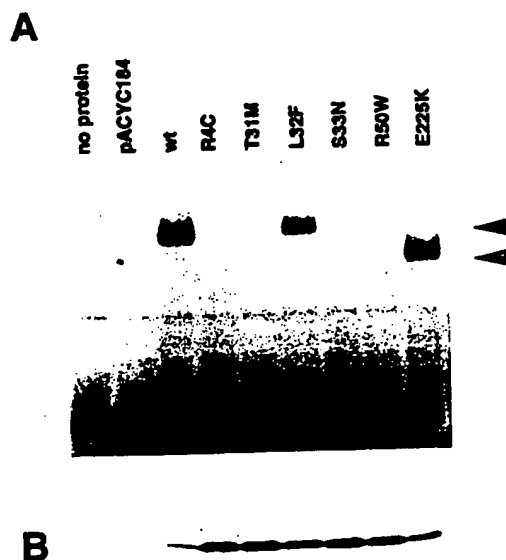


FIG. 4. (A) Gel retardation assay of nonrepressing OxyR mutants binding to the *oxyR-oxoS* promoters. Equal aliquots (1 μ l) of the extracts were incubated with a 100-bp *EcoRI-HindIII* fragment of pGSO40 (37) labeled at the *HindIII* site. The bound and unbound fragments were then separated on a low-ionic-strength polyacrylamide gel. The filled and stippled arrows indicate two different protein-DNA complexes formed. (B) Immunoblot of the extracts used in the gel retardation assay. Equal aliquots of the crude extracts were loaded in each lane. wt, wild type.



FIG. 5. Gel retardation assay of purified wild-type (wt) and A233V and E225K mutant proteins. Eight nanograms of the purified wild-type protein and 800 ng of the purified A233V and E225K mutant proteins were assayed. The filled and stippled arrows indicate the two different protein-DNA complexes observed.

mutants (Fig. 4 in reference 21 and data not shown). However, when DNA binding was assayed in a more sensitive gel retardation experiment, two of the mutants (L32F and E225K) showed a retarded band (Fig. 4A). The immunoblot in Fig. 4B shows that a full-length OxyR protein was present in all of the mutant extracts but that the mutant strains expressed higher levels of OxyR protein than the wild-type strain, in agreement with the nonrepressing phenotype of the mutants. Since equal amounts of total protein were assayed for all of the strains and the wild-type strain expressed significantly lower levels of OxyR, the relative binding affinity of the nonrepressing mutants is even lower than indicated by the intensity of the shifted band. The weak binding observed with mutants L32F and E225K correlates well with the weak transcriptional activity seen for these mutants *in vivo*. Only mutant R4C did not show any detectable binding to DNA but could slightly activate *oxyS* expression.

Interestingly, the E225K mutant led to a faster-migrating protein-DNA complex than the wild-type strain and mutant L32F. We had observed a similar faster-migrating complex in gel retardation experiments with extracts of a strain expressing the constitutively active A233V mutant described in the accompanying paper (21). To study the E225K and A233V proteins *in vitro*, the nonrepressing and constitutively active mutants were overexpressed and purified (21). When the gel retardation experiment was repeated with the pure proteins, it was obvious that both mutants showed protein-DNA complexes which had identical mobilities but migrated faster than the wild-type OxyR-DNA complex (Fig. 5). Both mutants also had significantly lower apparent affinities to the DNA than the wild-type protein, since a much larger amount of the protein (about 100-fold) was needed to obtain a visible retarded band. The increased mobility of the mutant protein-DNA complexes was not a function of the elevated protein concentrations needed to see binding, since the mobility of the wild-type OxyR-DNA complex was not altered by higher concentrations of wild-type protein (data not shown).

Oligomerization states of wild-type OxyR and A233V, E225K, and C199S mutants. Since the altered mobility ob-

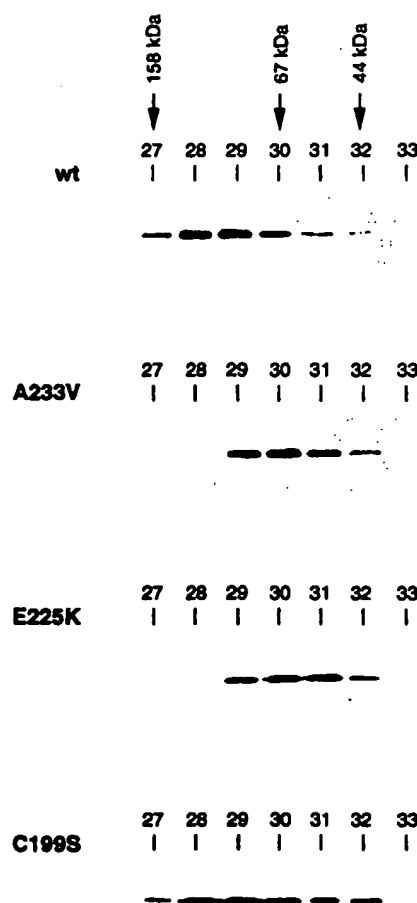


FIG. 6. Gel filtration analysis of wild-type (wt) OxyR and A233V, E225K, and C199S mutants. The purified proteins were run on a Superose 12 column, and the fractions were analyzed by immunoblots. The numbers denote the fraction numbers. The elution positions of the peak fractions for the standard proteins gamma globulin (158 kDa), bovine serum albumin (67 kDa), and ovalbumin (44 kDa) are indicated by the arrows.

served with the A233K and E225K protein-DNA complexes could be due to a difference in oligomerization, we examined the properties of the purified wild-type and A233V and E225K mutant proteins on a gel filtration column. Previous cross-linking experiments suggested that OxyR is a dimer in solution (35); however, several observations about the OxyR binding sites, such as the twofold dyad symmetry of the OxyR binding motif and the contacts made by OxyR in four adjacent major grooves, suggested that OxyR may act as a tetramer (37). The purified wild-type, A233V, and E225K mutant proteins were loaded on a Superose 12 gel filtration column, and the fractions were analyzed by immunoblots (Fig. 6). The wild-type protein eluted predominantly in fractions 28 to 30, with a peak between fractions 28 and 29. Assuming a globular conformation, this elution profile could correspond to a tetramer of the 34.4-kDa OxyR protein (137.6 kDa). The A233V and E225K mutant proteins eluted in fractions 29 to 31 with a peak at fraction 30, coinciding with the peak for the 67-kDa standard protein and suggesting that the A233V and E225K proteins are

TABLE 3. Galactokinase activities of *oxyR*⁺ wild-type (GSO27) and Δ *oxyR::kan* deletion (GSO7) strains encoding an *oxyR-galK* fusion and carrying the nonrepressing mutants on pACYC184

Protein	galK activity (U) ^a		Fold derepression ^b
	<i>oxyR</i> ⁺ strain	Δ <i>oxyR::kan</i> strain	
Vector	0.8 \pm 0.4	6.9 \pm 1.6	1
Wild type	0.6 \pm 0.1	0.5 \pm 0.5	0.8
R4C	5.7 \pm 1.7	5.7 \pm 1.0	7.1
T31M	6.9 \pm 0.4	6.8 \pm 0.7	8.6
L32F	4.3 \pm 1.1	5.7 \pm 0.1	5.4
S33N	5.2 \pm 1.7	5.7 \pm 0.2	6.5
R50W	4.1 \pm 1.5	7.8 \pm 1.2	5.1
E225K	1.1 \pm 0.8	3.5 \pm 0.2	1.4

^a The units of galactokinase activity were calculated as described previously (40). The average from two separate assays is given with the standard deviation.

^b The fold derepression was calculated by dividing the units of galK activity in the *oxyR*⁺ strains carrying the *oxyR* mutants by the units of activity in the *oxyR*⁺ strain carrying the vector (0.8 U).

dimers (68.8 kDa). These data are consistent with the observation that the A233V and E225K mutants led to a faster-migrating protein-DNA complex in the gel retardation assay and may bind to DNA as dimers.

Since wild-type OxyR purified in the absence of reducing agents is oxidized, we also examined the size of the wild-type protein when the sizing column was loaded and eluted in the presence of 100 mM dithiothreitol. The elution profile observed under reducing conditions was almost identical to the profile seen in the absence of dithiothreitol (data not shown). We also examined the size of the noninducible C199S mutant, which appears to be locked in the reduced conformation (21). The C199S protein eluted over a greater range of fractions than the wild-type protein, but much of the protein appears to be tetrameric. Therefore, since both oxidized and reduced wild-type OxyR can be described as tetramers and both the tetrameric wild-type protein and the dimeric E225K mutant are sensitive to oxidation, our results suggest that oxidation of the OxyR protein does not influence multimerization.

trans dominance of nonrepressing mutants. Having observed that OxyR is oligomeric, we tested whether the nonrepressing mutant proteins exhibit a negative *trans*-dominant phenotype and could inhibit the activity of the wild-type protein by forming inactive heteromultimers *in vivo*. We therefore transformed the plasmids encoding the nonrepressing mutants into a wild-type *oxyR* strain carrying the *oxyR-galK* fusion (GSO27) and compared the levels of galK expression with that in the original Δ *oxyR::kan* deletion (GSO7) background. As seen during the mutant screen, the vector-control strain and all of the nonrepressing mutants gave rise to red colonies in the Δ *oxyR::kan* background. In the *oxyR*⁺ background, the vector-control strain gave white colonies since the chromosomally encoded OxyR protein could repress the *oxyR-galK* fusion. In contrast, the colonies for the R4C, T31M, L32F, S33N, and R50W mutants were red, showing that the chromosomally encoded wild-type protein could not repress the fusion in the presence of these mutants. The colonies for the *oxyR*⁺ strain carrying E225K, however, were white, suggesting that the wild-type protein can still act as a repressor in the presence of this mutant.

We then assayed the levels of galactokinase activity in the *oxyR*⁺ and Δ *oxyR::kan* strains in a quantitative assay (Table 3). In the Δ *oxyR::kan* deletion background, as expected, all of the mutants had levels of galactokinase activity comparable to that of the vector-control strain. In the *oxyR*⁺ background, all mu-

tants except E225K had elevated levels of galactokinase activity and showed a nonrepressing phenotype. Therefore, the R4C, T31M, L32F, S33N, and R50W mutants are *trans* dominant and are able to "poison" the wild-type activity. The wild-type protein could still repress *oxyR* expression in the E225K mutant strain, suggesting that no mixed, inactive multimers are formed with E225K and revealing that this mutant is not *trans* dominant. This observation is consistent with our conclusion that the E225K mutation affects the multimerization of OxyR.

DISCUSSION

OxyR is a specific-DNA-binding protein which is able to activate as well as repress transcription of specific target genes. Here we used random mutagenesis to define regions of OxyR involved in DNA binding. We screened for mutants unable to repress an *oxyR-galK* fusion and identified six mutants which had elevated levels of *oxyR-galK* expression and exhibited decreased DNA binding in mobility shift and DNase I footprinting assays. The decreased-binding mutants also showed increased sensitivity to oxidants and decreased expression of the OxyR-activated *oxyS* gene in vivo, showing that OxyR-DNA binding is required for both activation and repression of the target genes.

The HTH motif represents a DNA binding domain of OxyR. Five of the six mutations (R4C, T31M, L32F, S33N, and R50W) mapped within or near the HTH motif which is conserved among the LysR family members. The decreased-binding phenotype of the nonrepressing mutants is consistent with the conclusion that the HTH region (residues 6 to 66) corresponds to the DNA binding domain in the LysR-type proteins (28). The R4C, S33N, and R50W mutations in OxyR affect amino acids that show a high degree of conservation among the LysR family members. Since many LysR family members bind to sequences that contain the very generic T-N₁₁-A motif (18), we propose that some of the highly conserved amino acids contact these conserved base pairs, while contacts by less highly conserved amino acids in the HTH domain provide specificity for the individual regulators. Future cross-linking experiments between the LysR-type proteins and the corresponding binding sites could test this hypothesis.

For OxyR, a region near the C terminus may also be critical for binding, since a protein with 22 amino acids truncated was not able to repress OxyR expression. A deletion of the C-terminal eight amino acids of the NahR protein also results in a loss of DNA binding (29). In contrast, the MetR protein tolerates substantial C-terminal deletions (38), suggesting that the C terminus is critical for DNA binding by only a subclass of LysR family members.

A C-terminal region of OxyR involved in tetramerization. One mutation (E225K) causing the nonrepressing phenotype did not map to the HTH motif but still caused decreased DNA binding. Since both the nonrepressing E225K mutant and the constitutively active A233V mutant led to faster-migrating protein-DNA complexes than wild-type OxyR in a gel retardation assay, we examined the sizes of these mutant proteins on gel filtration columns. While the oxidized wild-type OxyR protein was primarily tetrameric in solution, the E225K and A233V mutants appeared to be dimeric. This observation indicates that the E225K and A233V mutants are defective in multimerization and that the amino acids around positions 225 to 233 are directly or indirectly involved in tetramerization. Consistent with this interpretation, we found that the E225K mutant, unlike the other nonrepressing mutants, was not *trans* dominant.

The purified E225K and A233V mutants are similar in their abilities to bind DNA and induce expression of *oxyS* in vitro (21); however, several in vivo characteristics of these mutants are distinct. Both mutant proteins show weak binding in vitro, but E225K does not repress *oxyR* expression in vivo, while A233V is an efficient repressor. The E225K protein was also only a weak activator of *oxyS* expression under oxidizing conditions in vivo, while the A233V mutant was an extremely strong constitutive activator. Since the purified A233V protein had only a weak activity in vitro compared with its strong activation activity in vivo, it is likely that the A233V protein is modified during purification. Possibly some unknown conditions or factors present in intact cells act to stabilize the A233V mutant.

Our observation that the oxidized wild-type OxyR protein is a tetramer is consistent with the previous finding that the OxyR protein binds to four adjacent major grooves of the DNA helix (37). The twofold dyad symmetry of the OxyR binding motif (ATAGxtxxxCTATxxxxxxATAGxtxxxCTAT) suggests that the tetrameric OxyR protein may exist as a dimer of dimers (37). This would imply the presence of two multimerization domains in OxyR, one involved in dimerization and the other required for the tetramerization of two dimers. The E225K and A233V mutations could conceivably affect either the dimerization domain (resulting in dimers via the tetramerization domain) or the tetramerization domain (resulting in dimers via the dimerization domain). We cannot unambiguously distinguish between these possibilities, but we propose that the two OxyR mutations affect a putative tetramerization domain, since the C terminus seems to be dispensable for dimeric but not tetrameric LysR family members. We did not identify any mutations affecting the second multimerization domain. Possibly these mutations lead to an unstable form of the protein, or alternatively, the mutants are still *trans* dominant.

The NahR, TrpI, and CysB members of the LysR family have also been shown to be tetramers in solution (14, 26, 29), while other LysR proteins, such as MetR, NodD3, and Nac, appear to be dimers (17, 19, 25). The CysB protein has also recently been shown to bind DNA as a tetramer (20). It is interesting that for OxyR and possibly for other tetrameric LysR family members, the C-terminal domain is critical for DNA binding and the protein binds to approximately 45 bp. In contrast, for dimeric MetR, the C terminus seems to be dispensable for DNA binding and the protein binds to approximately 25 bp (reference 24 and references therein; 38). These differences suggest that there may be at least two different classes of LysR proteins, those which are able to bind and activate as dimers and others which are able to function only as tetramers.

The finding that OxyR exists as a multimer raises the possibility that the oligomerization state of OxyR might be regulated as a function of oxidation and reduction, similar to the case for the oxygen-sensitive transcriptional regulator FNR (23). We do not favor this mechanism for regulating the activity of OxyR, since the elution profile of wild-type OxyR under reducing conditions (data not shown) is identical to the elution profile of the oxidized protein. The inactive C199S mutant, which appears to be locked in the reduced state (21) and shows a protein-DNA shift similar to that with the oxidized wild-type protein (data not shown), can also be described as a tetramer. In addition, the dimeric A233V mutant is constitutively active, and the dimeric E225K protein is still responsive to hydrogen peroxide in vivo. Future studies of how the OxyR subunits are arranged, how the subunit contacts are changed upon oxidation, and how many subunits need to be oxidized in order to

induce transcription should give further insights into how the OxyR protein is activated by oxidation.

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